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RESEARCH ARTICLE

Adaptive Immunity

Cyclophilin D Regulates Antiviral CD8$^+$ T Cell Survival in a Cell-Extrinsic Manner

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ABSTRACT

CD8$^+$ T cell–mediated immunity is critical for host defense against viruses and requires mitochondria-mediated type I IFN (IFN-I) signaling for optimal protection. Cyclophilin D (CypD) is a mitochondrial matrix protein that modulates the mitochondrial permeability transition pore, but its role in IFN-I signaling and CD8$^+$ T cell responses to viral infection has not been previously explored. In this study, we demonstrate that CypD plays a critical extrinsic role in the survival of Ag-specific CD8$^+$ T cell following acute viral infection with lymphocytic choriomeningitis virus in mice. CypD deficiency resulted in reduced IFN-I and increased CD8$^+$ T cell death, resulting in a reduced antiviral CD8$^+$ T cell response. In addition, CypD deficiency was associated with an increase in pathogen burden at an early time-point following infection. Furthermore, our data demonstrate that transfer of wild-type macrophages (expressing CypD) to CypD-deficient mice can partially restore CD8$^+$ T cell responses. These results establish that CypD plays an extrinsic role in regulating optimal effector CD8$^+$ T cell responses to viral infection. Furthermore, this suggests that, under certain circumstances, inhibition of CypD function may have a detrimental impact on the host's ability to respond to viral infection. *ImmunoHorizons*, 2020, 4: 217–230.

INTRODUCTION

Cyclophilin D (CypD) is a member of cyclophilin protein family, which is encoded by the peptidyl-prolyl isomerase F (*Ppif*) gene and has peptidyl-prolyl-*cis-trans*-isomerase activity (1–4). CypD is located within the mitochondrial matrix and plays a critical modulatory role in the mitochondria permeability transition pore (MPTP) (1, 2). Various stresses can trigger a conformational

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S.A.C. and M.J.R. designed experiments; S.A.C., J.D., S.F.V., and R.D.P. performed experiments; E.T., A.L., and M.D. provided reagents; S.A.C. drafted the manuscript; all authors reviewed the manuscript.

Abbreviations used in this article: BMDM, bone marrow–derived macrophage; CypD, cyclophilin D; DC, dendritic cell; FLICA, fluorescent inhibitor of caspase; gMFI, geometric mean fluorescence intensity; IFN-I, type I IFN; LCMV, lymphocytic choriomeningitis virus; LCMV-Arm, LCMV Armstrong strain; MHCII, MHC class II; MPTP, mitochondria permeability transition pore; *Ppif*, peptidyl-prolyl isomerase F; WT, wild-type.

change of the MPTP, a transition that is facilitated by CypD, converting the pore to an open state (5). Upon pore opening, the mitochondria swell, causing membrane rupture and ultimately cell death via apoptosis or necrosis (5). CypD-mediated cell death has been heavily implicated in pathologies of the liver, heart, and lungs, and thus identification of drugs inhibiting CypD function, such as cyclosporine A, have drawn interest as potential therapies (5). Although CypD has recently been shown to be an intrinsic negative regulator of $CD8^+$ T cell responses to intracellular *Mycobacterium tuberculosis* bacterial infection (6), its role in antiviral immunity is currently unknown.

Cytotoxic $CD8^+$ T cells are critical for the control and elimination of intracellular pathogens such as viruses. To become fully activated, a naive $CD8^+$ T cell must receive three signals. The first signal (signal 1), requires the interaction between an Ag-specific $CD8^+$ T cell bearing the correct TCR and an APC such as a dendritic cell (DC) presenting cognate Ag on MHC class I (7–11). The second signal (signal 2) comprises the interaction of costimulatory molecules present on mature DCs such as CD80/CD86 and CD28 on the T cell (10, 12–15). Finally, inflammatory cytokines present at the time of activation, such as type I IFN (IFN-I), provide the third signal (signal 3) and are required for optimal $CD8^+$ T cell proliferation, accumulation, and generation of an effective $CD8^+$ T cell effector response (16–23). Upon receiving these three signals, the naive Ag-specific $CD8^+$ T cell undergoes clonal expansion and acquires the ability to produce effector cytokines (such as IFN-γ and TNF-α) and cytolytic molecules to control the replicating pathogen (24–32).

Given the critical role of CypD in mitochondrial integrity and the recent demonstration of CypD's role in T cell responses to *M. tuberculosis* bacterial infection (6) in which it acts in a cell-intrinsic manner to restrain T cell expansion, we asked whether deficiency in CypD impacts the $CD8^+$ T cell response to acute virus infection. In this study, we demonstrate that CypD plays an important cell-extrinsic role in promoting Ag-specific $CD8^+$ T cell survival. In addition, we observed that transfer of CypD-expressing macrophages in CypD-deficient hosts can partially rescue Ag-specific $CD8^+$ T cell numbers. This establishes a novel T cell–extrinsic role for CypD in antiviral immunity and highlights the potential adverse effects of administering CypD inhibitory drugs, such as cyclosporine A, as host immunity to certain viruses may be compromised.

MATERIALS AND METHODS

Mice and virus

C57BL/6 wild-type (WT), CypD-deficient (*Ppif*$^{-/-}$), and WT P14 TCR–transgenic (specific for lymphocytic choriomeningitis virus [LCMV]–derived GP_{33-41} epitope) (33) mice were bred at McGill University, and both male and female mice were used at 6–8 wk of age. LCMV Armstrong (LCMV-Arm) strain was kindly provided by John Harty (The University of Iowa) and propagated as described (19, 34, 35). Mice were infected with 2×10^5 PFU (injection i.p. route) and housed at the appropriate biosafety level, and the morbidity was monitored at indicated time points.

Peptides, MHC class I tetramer, flow cytometry Abs, proliferation, and cell death assays

Peptides were synthesized by either Bio Basic Canada ($GP_{276-286}$ [SGVENPGGYCL], $NP_{396-404}$ [FQPQNGQFI]) or Bio-Synthesis (GP_{33-41} [KAVYNFATM]). H-$2D^b$ MHC class I tetramers (D^b $NP_{396-404}$, D^b GP_{33-41}, or D^b $GP_{276-286}$) were prepared as previously described (36). The following Abs were used in an appropriate combination of fluorochromes: CD3 (clone 17A2; eBioscience), CD8α (clone 53-6.7; BioLegend), CD11a (clone M17/4; BioLegend), CD11b (clone M1/70; eBioscience), CD11c (clone N418; eBioscience), CD19 (clone MB19-1; eBioscience), CD40 (clone 1C10; eBioscience), CD80 (clone 16-10A1; eBioscience), CD86 (clone GL1; eBioscience), F4/80 (clone Bm8; eBioscience), IFN-γ (clone XMG1.2; eBioscience), MHC class II (MHCII) (clone M5/114.15.2; eBioscience), NK1.1 (clone PK136; eBioscience), TNF-α (clone MP6-XT22; BioLegend), and Ki67 (clone 16A8; BioLegend). Proliferation and cell death assays were performed according to the specifications of the manufacturer: Phase-Flow BrdU Cell Proliferation Kit (BioLegend), Vybrant FAM Caspase-3 and -7 Assay Kit (Molecular Probes), and FITC Annexin V apoptosis Detection Kit II (BD Biosciences).

Adoptive transfer and quantification of $CD8^+$ T cells

WT congenically marked (Thy1.1) P14 TCR–transgenic $CD8^+$ T cells were obtained from the peripheral blood of young naive P14 female mice, and 5000 cells were injected i.v. into either naive WT (Thy1.2) or naive *Ppif*$^{-/-}$ (Thy1.2)–recipient mice. Quantification of endogenous response was done in the absence of adoptive transfer. Mice were infected with LCMV-Arm the following day (2×10^5 PFU i.p.). Spleens were isolated on day 8 postinfection and processed into single-cell suspensions. RBCs were lysed with ACK buffer (ACK buffer: 150 mM NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na_2EDTA, dissolved in H_2O and adjusted to [pH 7.2–7.4]), and lymphocytes were stained with indicated Abs then fixed with IC Fixation Buffer (eBioscience). Total $CD8^+$ T cells were identified with CD8α staining, total endogenous LCMV-specific responses were identified with CD8α CD11a staining (37, 38), endogenous LCMV-specific $CD8^+$ T cells were identified by H-$2D^b$ MHC class I tetramer (D^b $NP_{396-404}$, D^b GP_{33-41}, or D^b $GP_{276-286}$) CD8α staining or WT P14 TCR–transgenic $CD8^+$ T cells were identified with Thy1.1 and CD8α staining. Samples were analyzed with a BD LSRFortessa Flow Cytometer (BD Biosciences) and FlowJo software (Tree Star).

Ex vivo stimulation and intracellular cytokine staining

On day 8 post–LCMV-Arm infection, spleens were harvested and processed into single-cell suspensions. RBCs were lysed with ACK buffer and samples were stimulated for 5.5 h at 37°C with 5% CO_2 in the presence or absence of peptide ($NP_{396-404}$, GP_{33-41}, or $GP_{276-286}$) and brefeldin A (BioLegend). The frequency of $CD8^+$ T cells capable of producing cytokine after stimulation with indicated peptide was determined with intracellular staining for

cytokines (IFN-γ, TNF-α) stained in Perm/Wash Buffer (eBioscience). Samples were analyzed with a BD LSRFortessa Flow Cytometer (BD Biosciences) and FlowJo software (Tree Star).

Virus quantitation

On day 3 postinfection, livers were harvested and homogenized, and the virus was quantitated using Vero cell plaque assay as previously described (19, 34, 35).

IFN-I quantification

On day 3 postinfection, serum was collected via cardiac puncture from WT and *Ppif*$^{-/-}$ mice and levels of total active IFN-I (both IFN-α and IFN-β) were assessed using cultured B16-blue IFN-α/-β reporter cell line (InvivoGen), according to the specifications of the manufacturer. Briefly, 25 μl of samples was incubated with 175 μl of B16 cells (850,000 cells/ml) at 37°C and 5% CO_2 overnight in a 96-well plate. Following, 25 μl of B16 cell supernatant was incubated at 37°C and 5% CO_2 with 175 μl of QUANTI-Blue medium (InvivoGen) and OD_{620} readings were taken beginning at 1 h postincubation.

B16 cell culture

B16 cells were maintained in RPMI 1640 enriched with 10% FBS, 2 mM L-glutamine, and 100 U/ml of penicillin/streptomycin. One hundred micrograms per milliliter of Zeocin and 200 μg/ml of Normocin (InvivoGen) were added to the cell culture media for all subsequent passages and assays.

Bone marrow–derived macrophages and rescue experiment

Bone marrow was extracted from the femur and tibia of WT and *Ppif*$^{-/-}$ mice. Macrophages were differentiated from each mouse strain for 1 wk in bone marrow–derived macrophage (BMDM) media (BMDM media: RPMI 1640 supplemented with 10% FBS, 1% penicillin-streptomycin, 1% L-glutamine, 1% nonessential amino acids, 1% essential amino acids, 1% sodium pyruvate, 2% HEPES, 0.14% sodium hydroxide) containing 30% L929 cell culture supernatant (L929 supernatant contains M-CSF growth factor for macrophage differentiation). For rescue experiments, 1×10^6 BMDM were injected i.v. into naive *Ppif*$^{-/-}$–recipient mice. Mice were infected with LCMV-Arm the following day (2×10^5 PFU i.p.), and on day 8 postinfection, splenocytes were isolated and CD8$^+$ T cell analysis was performed as described above.

Statistical analysis

Data were analyzed using GraphPad Prism6 software. Unpaired Student *t* tests were performed and *p* values < 0.05 were considered statistically significant. The *p* values are represented as follows: $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Study approval

All animal procedures were carried out in accordance with the Canadian Council on Animal Care and were approved by the McGill University Animal Care Committee (Protocol no. 7554).

RESULTS

CD8$^+$ T cell numbers are equivalent in naive WT and CypD-deficient mice

Before investigating whether CypD deficiency has an impact on CD8$^+$ T cell antiviral immunity, we first examined total spleen cellularity and CD8$^+$ T cell numbers from naive C57BL/6 WT and *Ppif*$^{-/-}$ (CypD-deficient) mice. We observed no differences in total spleen cellularity (Fig. 1A) and no reduction in frequencies (Fig. 1B) and total numbers (Fig. 1C) of CD8$^+$ T cells in naive *Ppif*$^{-/-}$ mice compared with WT mice. Together, these results established there is no reduction in baseline CD8$^+$ T cell numbers in naive CypD-deficient mice, demonstrating that at steady-state, CypD deficiency does not affect total CD8$^+$ T cell numbers. Thus, this model can be used to study the role of CypD in CD8$^+$ T cell responses to viral infection.

CypD is required for optimal antiviral CD8$^+$ T cell responses

To determine whether CypD deficiency impacts antiviral immune responses, we infected naive (WT) and *Ppif*$^{-/-}$ mice with LCMV-Arm and assessed morbidity and viral load. LCMV-Arm, an enveloped ambisense RNA virus that is a prototypic virus in the *Arenaviridae* family, is a natural mouse pathogen that establishes an acute and typically asymptomatic viral infection in mice (39) that is controlled by a robust CD8$^+$ T cell response (40). As expected following infection with LCMV-Arm, C57BL/6 WT mice exhibited minimal weight loss that returned to preinfection weight levels on day 5 postinfection (Fig. 2A). In contrast, *Ppif*$^{-/-}$ mice exhibited a slight but significant increase in morbidity with significant weight loss that did not return to preinfection weight levels during the 8-d time course (Fig. 2A). Importantly, the increased morbidity correlated with compromised viral control, as demonstrated by a significant increase in viral titers in the livers from day 3 post–LCMV-Arm–infected *Ppif*$^{-/-}$ mice compared with WT mice (Fig. 2B, 5.8-fold). Together, these results suggested that deficiency in CypD compromises host responses, leading to a higher viral burden at an early time-point and increased morbidity following challenge with a virus that typically establishes a relatively asymptomatic infection.

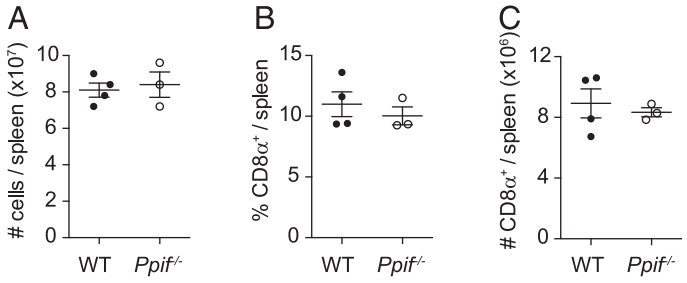

FIGURE 1. CypD deficiency does not impact CD8$^+$ T cell numbers at steady-state.

Splenocytes isolated from naive WT or *Ppif*$^{-/-}$ mice. (**A**) Overall spleen cellularity. (**B**) Frequency of CD8α$^+$ T cells. (**C**) Total number of CD8α$^+$ T cells in the spleen. Data analyzed by two-tailed, unpaired Student *t* test. Error bars represent SEM.

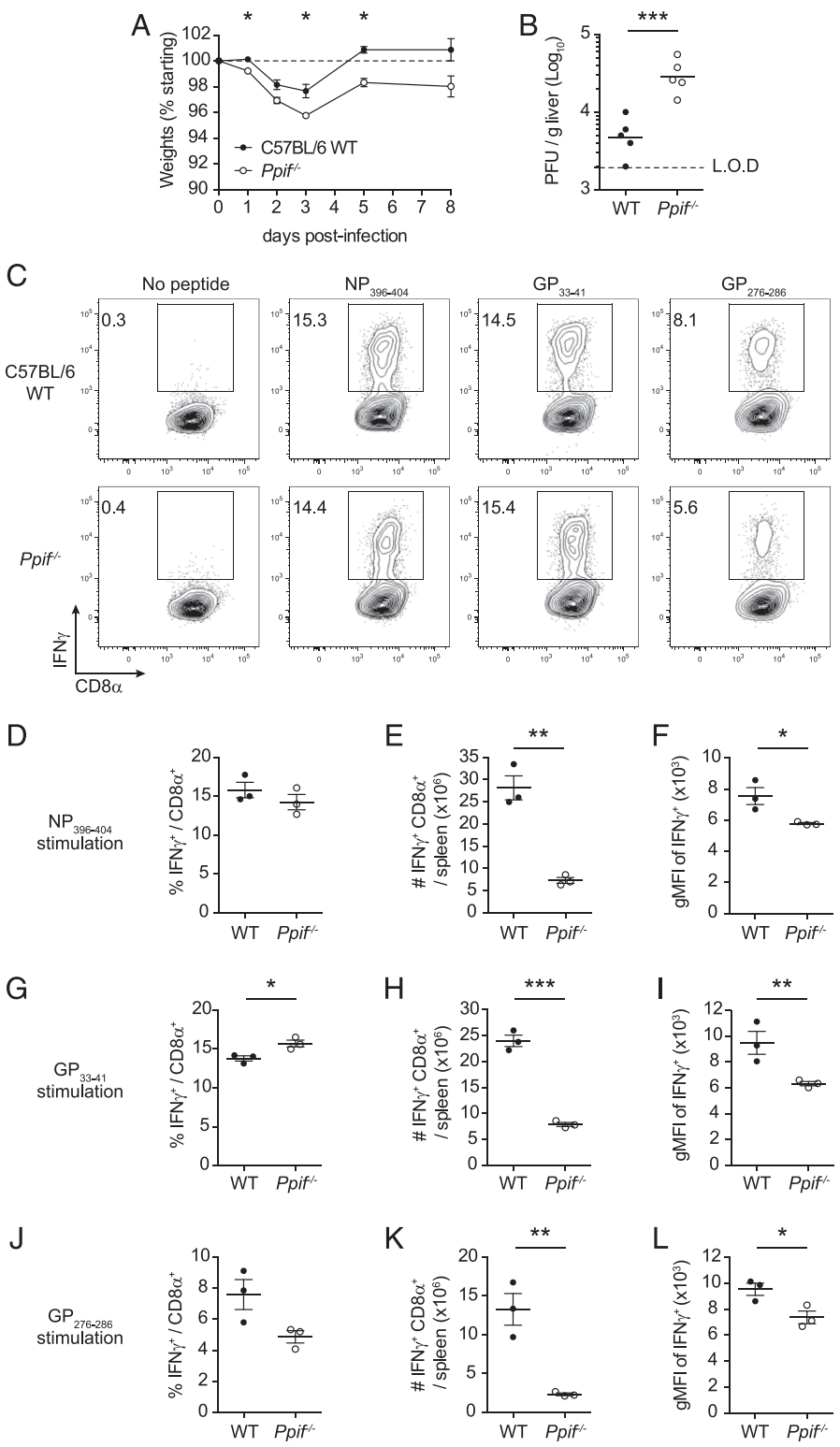

FIGURE 2. CypD deficiency impacts CD8+ T cell antiviral immunity.

(**A**) C57BL/6 WT or *Ppif*$^{-/-}$ morbidity curves post–LCMV-Arm infection. Mouse weights were plotted as a percentage of weight at the start of the experiment. (**B**) Viral burden in the liver from WT or *Ppif*$^{-/-}$ day 3 post–LCMV-Arm–infected mice. WT or *Ppif*$^{-/-}$ mice were infected with LCMV-Arm (2×10^5 PFU i.p.), and on day 8 postinfection, CD8$^+$ T cell analysis was performed. (**C**) Gated on CD8α^+ T cells, illustrated are representative flow cytometry plots of ex vivo stimulation of splenocytes stimulated with peptide ($NP_{396-404}$, GP_{33-41}, or $GP_{276-286}$). The **(Continued)**

CD8$^+$ T cells are important for limiting LCMV-Arm infection–associated morbidity and for controlling viral burden, so we next assessed whether deficiency in CypD impacts Ag-specific antiviral CD8$^+$ T cell responses. One advantage of using LCMV-Arm as a model pathogen is that multiple H-2D^b–restricted virus-specific CD8$^+$ T cell epitopes have been identified in C57BL/6 WT mice with $NP_{396–404}$, $GP_{33–41}$, and $GP_{276–286}$ shown to be the three immunodominant epitopes (41–44). Splenocytes isolated from day 8 LCMV-Arm–infected WT and *Ppif*$^{-/-}$ mice were stimulated in the presence of LCMV-derived peptides ($NP_{396–404}$, $GP_{33–41}$, or $GP_{276–286}$) and brefeldin A, and the frequency of CD8$^+$ T cells capable of producing IFN-γ was determined via intracellular staining (Fig. 2C). Following ex vivo stimulation with $NP_{396–404}$ peptide, we observed no significant change in the frequencies of CD8$^+$ T cells capable of producing the cytokine IFN-γ between WT and *Ppif*$^{-/-}$ mice (Fig. 2C, 2D). However, total number of IFN-γ–producing $NP_{396–404}$–specific CD8$^+$ T cells were significantly lower in *Ppif*$^{-/-}$ mice compared with WT mice (Fig. 2E, 3.9-fold). In addition, the amount of IFN-γ produced on a per-cell basis (as measured by geometric mean fluorescence intensity [gMFI] for IFN-γ) after ex vivo stimulation was also significantly reduced in cells isolated from *Ppif*$^{-/-}$ mice compared with WT mice (Fig. 2F). Ex vivo stimulation of splenocytes with $GP_{33–41}$ peptide revealed that *Ppif*$^{-/-}$ mice harbored slightly higher frequencies of CD8$^+$ T cells capable of producing IFN-γ (Fig. 2G). Nevertheless, we observed a substantial decrease in the overall magnitude of the IFN-γ producing $GP_{33–41}$–specific CD8$^+$ T cell response (Fig. 2H, 3.0-fold) in *Ppif*$^{-/-}$ mice compared with WT mice, as well as a reduction in the amount of IFN-γ produced on a per-cell basis in $GP_{33–41}$–specific CD8$^+$ T cells (Fig. 2I). Similarly, following ex vivo $GP_{276–286}$ peptide stimulation, we observed no significant reduction in the frequency of CD8$^+$ T cells able to produce IFN-γ (Fig. 2J) but a significant reduction in the total numbers of IFN-γ–producing $GP_{276–286}$–specific CD8$^+$ T cells (Fig. 2K, 5.7-fold) and a significant decrease in the expression of IFN-γ produced on a per-cell basis (Fig. 2L) in cells from *Ppif*$^{-/-}$ mice compared with WT mice. Together, these results demonstrated that deficiency in CypD is associated with a reduction in the overall number of CD8$^+$ T cells specific for the three LCMV immunodominant epitopes examined and a reduction in their capacity to express IFN-γ on a per-cell basis.

Intracellular cytokine staining analyses revealed reduced functional LCMV-specific CD8$^+$ T cells, but these assays cannot distinguish whether this is due to a reduction in the capacity of T cells to produce cytokines (e.g., exhaustion) versus impaired T cell expansion/survival in *Ppif*$^{-/-}$ mice. Thus, we further evaluated the magnitude of the LCMV-specific CD8$^+$ T cell response in WT and *Ppif*$^{-/-}$ mice using MHC class I tetramer reagents. Splenocytes were isolated from day 8 LCMV-Arm–infected WT and *Ppif*$^{-/-}$ mice and LCMV-Arm–specific CD8$^+$ T cells were enumerated with H-2D^b MHC class I tetramers (D^b $NP_{396–404}$, D^b $GP_{33–41}$, or D^b $GP_{276–286}$) (Fig. 3A). Although the frequencies of both D^b $NP_{396–404}$– and D^b $GP_{33–41}$–specific CD8$^+$ T cells were significantly increased in *Ppif*$^{-/-}$ mice (Fig. 3B, 3D, respectively), we observed a significant reduction in the total number of D^b $NP_{396–404}$– (Fig. 3C, 2-fold) and D^b $GP_{33–41}$ (Fig. 3E, 2.5-fold)–specific CD8$^+$ T cells compared with WT mice. The frequencies of D^b $GP_{276–286}$–specific CD8$^+$ T cells were similar in the WT and *Ppif*$^{-/-}$ mice (Fig. 3F); however, the total numbers of D^b $GP_{276–286}$–specific CD8$^+$ T cells were reduced in *Ppif*$^{-/-}$ mice (Fig. 3G, 4.1-fold). These results confirmed CypD also plays an important role in regulating the magnitude of expansion of three immunodominant LCMV-specific CD8$^+$ T cell populations.

CypD deficiency globally impacts antiviral CD8$^+$ T cell accumulation

Our data highlighted that although the numbers of immunodominant epitope-specific CD8$^+$ T cells was decreased, the overall frequencies of Ag-specific cells within the CD8$^+$ T cell compartment was unchanged following infection. Thus, our data suggest that CypD deficiency broadly affects the accumulation of all Ag-specific CD8$^+$ T cells following viral infection, resulting in a decrease in number without affecting the frequency of cells within the CD8$^+$ T cell compartment responding to each epitope. To evaluate whether the absence of CypD globally impacts CD8$^+$ T cell responses to LCMV infection, regardless of their Ag specificity, we enumerated the total number of Ag-experienced CD8$^+$ T cells responding to LCMV-Arm infection using a surrogate marker approach. Previous studies have established that surrogate markers allow for the analysis of the total number of all Ag-experienced T cells responding to infection without a priori knowledge of Ag specificity (37, 38, 45, 46). For CD8$^+$ T cells, Ag-experienced cells can be identified by the downregulation of CD8α (CD8α^{lo}) and up regulation of CD11a (CD11a^{hi}), which enables evaluation of the total CD8$^+$ T cell response induced by infection (37, 38). Splenocytes were isolated from day 8 LCMV-Arm–infected WT and *Ppif*$^{-/-}$ mice, and total numbers of Ag-experienced

frequency of CD8α$^+$ T cells capable of producing IFN-γ after ex vivo stimulation with peptide is indicated in each plot. (**D**) Frequency of CD8α$^+$ T cells producing IFN-γ after $NP_{396–404}$ stimulation. (**E**) The total number of IFN-γ$^+$ $NP_{396–404}$–specific CD8α$^+$ T cells in the spleen after $NP_{396–404}$ stimulation. (**F**) gMFI of IFN-γ$^+$ CD8α$^+$ T cells after $NP_{396–404}$ stimulation. (**G**) Frequency of CD8α$^+$ T cells producing IFN-γ after $GP_{33–41}$ stimulation. (**H**) The total number of IFN-γ$^+$ $GP_{33–41}$–specific CD8α$^+$ T cells in the spleen after $GP_{33–41}$ stimulation. (**I**) gMFI of IFN-γ$^+$ CD8α$^+$ T cells after $GP_{33–41}$ stimulation. (**J**) Frequency of CD8α$^+$ T cells producing IFN-γ after $GP_{276–286}$ stimulation. (**K**) The total number of IFN-γ$^+$ $GP_{276–286}$–specific CD8α$^+$ T cells in the spleen after $GP_{276–286}$ stimulation. (**L**) gMFI of IFN-γ$^+$ CD8α$^+$ T cells after $GP_{276–286}$ stimulation. Data analyzed by two-tailed, unpaired Student *t* test. Error bars represent SEM. Data are representative of two similar and independent experiments (three to five mice per group). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. L.O.D, limit of detection.

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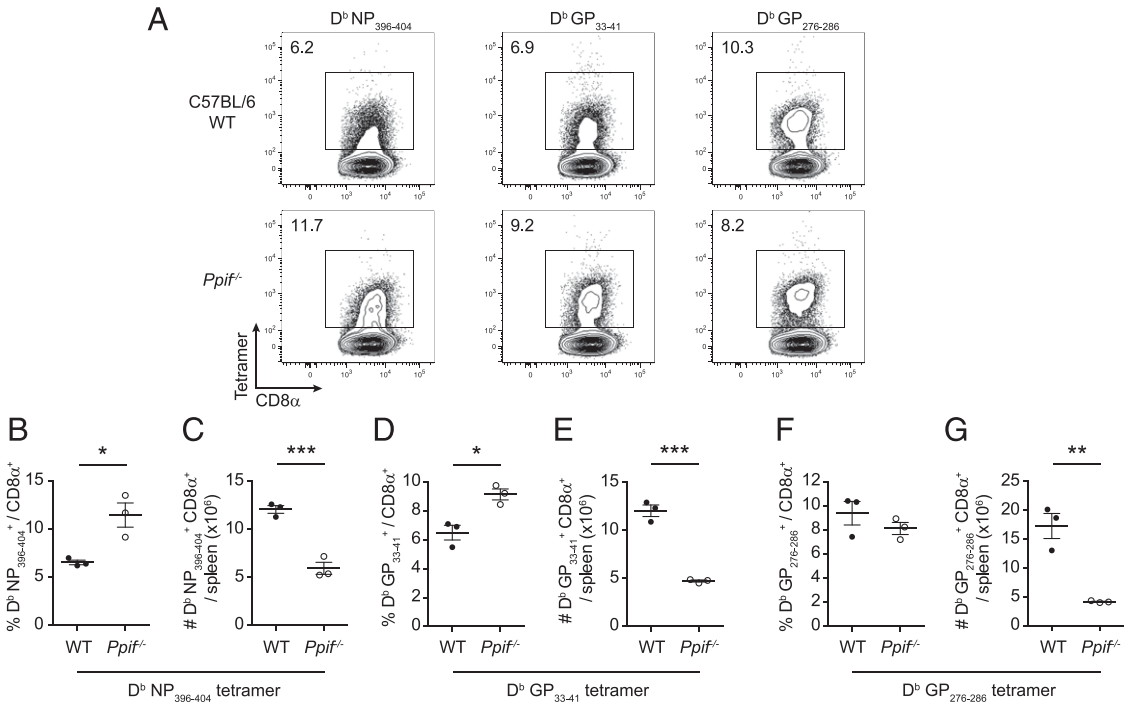

FIGURE 3. CypD deficiency impairs LCMV-specific CD8+ T cell responses.

WT or *Ppif*$^{-/-}$ mice were infected with LCMV-Arm (2×10^5 PFU i.p.), and on day 8 postinfection, CD8$^+$ T cell analysis was performed. (**A**) Illustrated are representative flow cytometry plots of MHC class I H-2D^b tetramer (D^b NP$_{396-404}$, D^b GP$_{33-41}$, or D^b GP$_{276-286}$) staining of CD8α$^+$ T cells in the spleen. The frequency of MHC class I H-2D^b tetramer$^+$ CD8α$^+$ T cells is indicated in each plot. (**B**) Frequency of D^b NP$_{396-404}$–specific CD8α$^+$ T cells. (**C**) Total number of D^b NP$_{396-404}$–specific CD8α$^+$ T cells in the spleen. (**D**) Frequency of D^b GP$_{33-41}$–specific CD8α$^+$ T cells. (**E**) Total number of D^b GP$_{33-41}$–specific CD8α$^+$ T cells in the spleen. (**F**) Frequency of D^b GP$_{276-286}$–specific CD8α$^+$ T cells. (**G**) Total number of D^b GP$_{276-286}$–specific CD8α$^+$ T cells in the spleen. Data analyzed by two-tailed, unpaired Student *t* test. Error bars represent SEM. Data are representative of two similar and independent experiments (three to five mice per group). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

LCMV-specific CD8$^+$ T cells were enumerated with CD8α and CD11a staining (Fig. 4A). In the bulk CD8$^+$ T cell population, we observed significant reductions in the frequencies (Fig. 4B) and total numbers (Fig. 4C, 3.5-fold) of CD8$^+$ T cells in *Ppif*$^{-/-}$ mice compared with WT mice following LCMV-Arm infection. Furthermore, based on CD8α^{lo} CD11a^{hi} expression patterns, we found that although the frequencies of Ag-experienced CD8$^+$ T cells were minimally lower (Fig. 4D), the total number of Ag-experienced CD8α^{lo} CD11a^{hi} CD8$^+$ T cells was significantly decreased (Fig. 4E, 3.6-fold) in *Ppif*$^{-/-}$ mice compared with WT mice following LCMV-Arm infection. The decreased cell numbers observed was attributed by a decrease in total spleen cellularity (Fig. 4F, 2.2-fold) and the reduction in CD8$^+$ T cell numbers was the primary contributor, representing ~70% of the overall reduction. Together, this suggests that CypD deficiency globally affects the accumulation of all Ag-specific CD8$^+$ T cells during the course of viral infection.

CD8$^+$ T cells from CypD-deficient mice exhibit reduced polyfunctionality

In addition to the magnitude (the number of cells responding) of the CD8$^+$ T cell response, the quality (diversity of function) of the responding T cells is also important for an effective antiviral immunity. Specifically, the production of multiple cytokines (polyfunctionality; i.e., the ability of a single cell to produce two or more cytokines) is associated with protective immune responses against various pathogens (47–51). Thus, we next examined whether deficiency in CypD affected CD8$^+$ T cell polyfunctionality. Splenocytes were isolated from day 8 LCMV-Arm–infected WT and *Ppif*$^{-/-}$ mice and stimulated in the presence of peptide (NP$_{396-404}$, GP$_{33-41}$, or GP$_{276-286}$) and brefeldin A, and the frequencies of stimulated CD8$^+$ T cells capable of producing both IFN-γ and TNF-α (i.e., double cytokine–producing cells) were determined by intracellular staining. *Ppif*$^{-/-}$ mice harbored significantly lower frequencies (Fig. 5A, 5B) and total numbers (Fig. 5C, 5.2-fold) of NP$_{396-404}$–specific CD8$^+$ T cells expressing both IFN-γ and TNF-α. In addition, we observed a significant decrease in the amount of TNF-α produced on a per-cell basis (as measured by TNF-α gMFI) from *Ppif*$^{-/-}$ cells compared with WT cells (Fig. 5D). Similar results were observed after ex vivo stimulation of splenocytes with either GP$_{33-41}$ or GP$_{276-286}$ peptide (Fig. 5E–J). Collectively, these results demonstrated that deficiency in CypD not only reduced the number of IFN-γ TNF-α double–producing LCMV-specific CD8$^+$

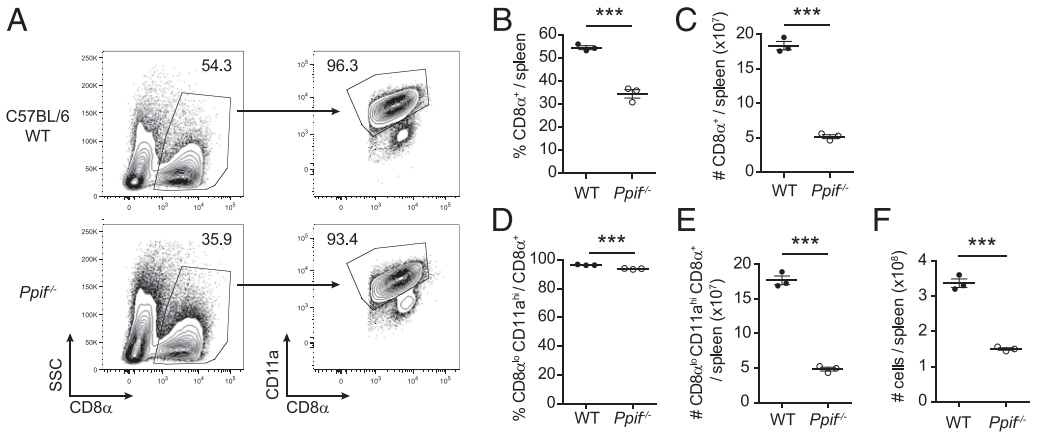

FIGURE 4. CypD deficiency impacts the accumulation of all Ag-experienced CD8$^+$ T cells.
WT or *Ppif*$^{-/-}$ mice were infected with LCMV-Arm (2×10^5 PFU i.p.), and on day 8 postinfection, CD8$^+$ T cell analysis was performed. (**A**) Representative flow cytometry plots of CD8α and CD11a expression on CD8α$^+$ T cells isolated from the spleen. The frequency of CD8α is indicated in the left plot, and the frequency of CD8α^{lo} CD11a^{hi} CD8α$^+$ T cells is indicated in the right plot. (**B**) Frequency of CD8α$^+$ T cells in the spleen. (**C**) Total number of CD8α$^+$ T cells in the spleen. (**D**) Frequency of CD8α^{lo}CD11a^{hi} Ag-experienced CD8α$^+$ T cells. (**E**) Total number of CD8α^{lo}CD11a^{hi} Ag-experienced CD8α$^+$ T cells in the spleen. (**F**) Overall spleen cellularity from WT or *Ppif*$^{-/-}$ day 8 post–LCMV-Arm–infected mice. Data analyzed by two-tailed, unpaired Student *t* test. Error bars represent SEM. Data are representative of two similar and independent experiments (three to five mice per group). ***$p < 0.001$.

T cells but also impacts their polyfunctionality on a per-cell basis, irrespective of the targeted viral epitope. Thus, CypD deficiency impaired the quality of CD8$^+$ T cells, demonstrating that both CD8$^+$ T cell accumulation and function may be compromised in the absence of CypD.

Cell-extrinsic factors contribute to CD8$^+$ T cell impairment, leading to decreased cell survival

Thus far, our results have demonstrated that CypD deficiency compromises CD8$^+$ T cell numbers and function. A recent study demonstrated that CypD elicits T cell–intrinsic effects to negatively regulate T cell expansion during mycobacterial infection (6). Because our data point to a positive role for CypD during acute viral infection, we next asked whether CypD plays this role in a T cell–intrinsic or –extrinsic manner. To address this, we used an adoptive transfer approach. Congenically marked WT TCR-transgenic P14 CD8$^+$ T cells (CD8$^+$ T cells specific for the LCMV-derived $GP_{33–41}$ epitope), which express CypD, were injected into either WT or *Ppif*$^{-/-}$–recipient mice. One day posttransfer, recipient mice were infected with LCMV-Arm, and WT P14 CD8$^+$ T cell expansion was examined on day 8 postinfection. We observed a significant reduction in the frequencies (Fig. 6A, 6B) and total numbers (Fig. 6C, 8.3-fold) of WT P14 CD8$^+$ T cells recovered in the spleens of *Ppif*$^{-/-}$ mice compared with WT mice on day 8 postinfection. Given that donor-derived P14 T cells express CypD, these data showed that the accumulation defect of LCMV-specific CD8$^+$ T cells in CypD-deficient mice is due, at least in part, to CD8$^+$ T cell–extrinsic factors.

Because IFN-Is (i.e., IFN-α and IFN-β) are important for CD8$^+$ T cell–mediated antiviral immunity, we reasoned that these inflammatory cytokines (i.e., signal 3) may be reduced in mice lacking CypD, potentially explaining the cell-extrinsic impairment in CD8$^+$ T cell accumulation following LCMV-Arm infection. To address this, we collected serum from WT and *Ppif*$^{-/-}$ mice at day 3 postinfection with LCMV-Arm and examined bioactive IFN-I levels with a reporter cell line. We observed a significant decrease in detectable bioactive IFN-Is in the serum of LCMV-Arm infected *Ppif*$^{-/-}$ mice compared with WT mice (Fig. 6D, 2.0-fold). IFN-I has been demonstrated to play a role in the accumulation of CD8$^+$ T cells in vivo by increasing cellular proliferation (20). Thus, using the same adoptive transfer approach, we examined cell proliferation of WT P14 CD8$^+$ T cells recovered in the spleens of WT and *Ppif*$^{-/-}$ mice 8 d post–LCMV-Arm infection. We assessed cell proliferation in two ways: first, we examined Ki67 expression, which is a marker of proliferating cells only expressed when cells enter the cell cycle (post–G0-phase), and we observed no differences in the frequency of Ki67$^+$ WT P14 CD8$^+$ T cells from spleens of WT and *Ppif*$^{-/-}$ mice 8 d postinfection (Fig. 6E, 6F). Second, we pulsed mice with BrdU on day 0 and maintained them on BrdU drinking water for 8 d. BrdU incorporates into cells with newly synthesized DNA during the S-phase of mitosis, and as such, more BrdU incorporation indicates increased cell proliferation. We observed no differences in the frequency of WT P14 CD8$^+$ T cells incorporating BrdU in spleens of WT and *Ppif*$^{-/-}$ mice 8 d post–LCMV-Arm infection (Fig. 6G, 6H). Together, these results demonstrated that the ability of CD8$^+$ T cells to enter cell cycle and to proliferate following infection are not regulated by CypD, although CypD deficiency does lead to a reduction in IFN-I production.

Given that our results showed no defect in cell proliferation, we next examined whether increased cell death was contributing

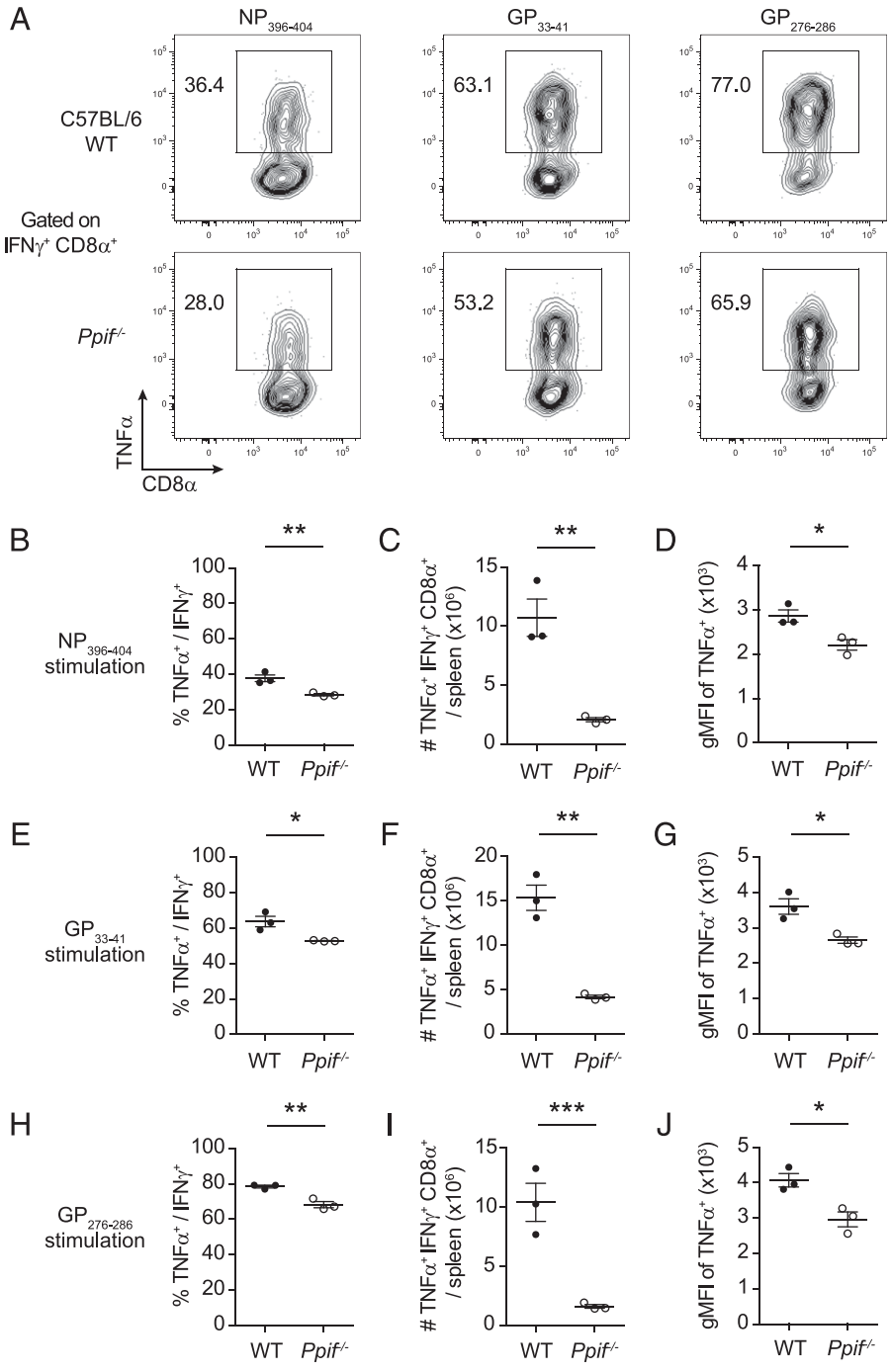

FIGURE 5. CD8+ T cell polyfunctionality is impaired in CypD-deficient mice.

C57BL/6 WT or *Ppif*$^{-/-}$ mice were infected with LCMV-Arm (2×10^5 PFU i.p.), and on day 8 postinfection, CD8+ T cell analysis was performed. (**A**) Gated on IFN-γ+ CD8α+ T cells, illustrated are representative flow cytometry plots following ex vivo stimulation of splenocytes stimulated in the presence of peptide ($NP_{396-404}$, GP_{33-41}, or $GP_{276-286}$). The frequency of CD8α+ T cells capable of producing both IFN-γ and TNF-α cytokines (i.e., double cytokine–producing cells) after stimulation is indicated in each plot. (**B**) Frequency of CD8α+ T cells producing IFN-γ and TNF-α cytokines after $NP_{396-404}$ stimulation. (**C**) The total number of IFN-γ+ TNF-α+ $NP_{396-404}$–specific CD8α+ T cells in the spleen after $NP_{396-404}$ stimulation. (**D**) gMFI of TNF-α+ CD8α+ T cells after $NP_{396-404}$ stimulation. (**E**) Frequency of CD8α+ T cells producing IFN-γ and TNF-α cytokines after GP_{33-41} stimulation. (**F**) The total number of IFN-γ+ TNF-α+ GP_{33-41}–specific CD8α+ T cells in the spleen after GP_{33-41} stimulation. (**G**) gMFI of TNF-α+ CD8α+ T cells after GP_{33-41} stimulation. (**H**) Frequencies of CD8α+ T cells producing IFN-γ and TNF-α cytokines after $GP_{276-286}$ **(Continued)**

to the reduction in $CD8^+$ T cell numbers observed. First, we examined cell death via Annexin V staining, which is used in conjunction with viability dye staining to determine the percentage of cells that are undergoing apoptosis. We observed significant increases in the frequency of WT P14 $CD8^+$ T cells undergoing apoptosis (Annexin V^+ $Zombie^-$) from *Ppif*$^{-/-}$ mice compared with WT mice 8 d post–LCMV-Arm infection (Fig. 6I, 6J). Next, we examined the activation of caspase enzymes using a Caspase-3/7 assay. The fluorescent inhibitor of caspases (FLICA) interacts with the enzymatic reactive center of activated caspase, thus directly measuring the amount of active caspase present. We observed a significant increase in the frequency of WT P14 $CD8^+$ T cells positive for FLICA from *Ppif*$^{-/-}$ mice compared with WT mice 8 d post–LCMV-Arm infection (Fig. 6K, 6L), indicating that the cells are expressing active caspases. Together, these results demonstrated that the reduction in WT P14 $CD8^+$ T cell numbers observed post–LCMV-Arm infection is attributed to an increase in apoptotic cells in *Ppif*$^{-/-}$ mice.

CypD deficiency impacts APCs

To assess which $CD8^+$ T cell–extrinsic factors are contributing to the $CD8^+$ T cell impairment in the absence of CypD, we first examined frequencies and numbers of DCs in LCMV-Arm–infected WT and *Ppif*$^{-/-}$ mice on day 2 postinfection (DCs identified as $CD3^-$, $CD19^-$, $NK1.1^-$ $CD11c^+$, $MHCII^+$ cells). We observed reductions in both the frequencies (Fig. 7A, 7B) and total numbers (Fig. 7C, 2.1-fold) of DCs in *Ppif*$^{-/-}$ mice compared with WT mice on day 2 postinfection. Although these data show that DC numbers are reduced following LCMV-Arm infection from mice lacking CypD, it has been demonstrated that overall DC numbers are not critical for the induction of an optimal $CD8^+$ T cell response following LCMV-Arm infection (52). Thus, this partial reduction in DC numbers is unlikely to be the key factor in the $CD8^+$ T cell impairment we observed in *Ppif*$^{-/-}$ mice. Moreover, the maturation and activation status of DCs, rather than total number, are the major drivers of T cell activation by DCs. Thus, we next evaluated whether DC activation and/or maturation is altered in LCMV-Arm–infected *Ppif*$^{-/-}$ mice compared with WT mice by assessing the expression of CD40, CD80, and CD86 on day 2 postinfection. CD40 is a costimulatory transmembrane protein that is expressed at low levels on resting DCs and CD80 and CD86 are costimulatory ligands expressed by activated APCs (53–58). We observed similar per-cell expression of CD40 by DCs recovered from day 2 post–LCMV-Arm–infected *Ppif*$^{-/-}$ mice compared with WT mice (Fig. 7D, 7E). In contrast, DCs in *Ppif*$^{-/-}$ mice expressed significantly more CD80 and CD86 protein on a per-cell basis (Fig. 7F–I). These results suggested that DC activation and maturation are unaffected (or enhanced) in *Ppif*$^{-/-}$ mice following LCMV-Arm infection, suggesting that other extrinsic factors may be contributing to the $CD8^+$ T cell impairment in mice deficient in CypD. These data are in line with our observations that the proportions of $CD8^+$ T cells that encounter Ag (as analyzed by the expression of surrogate markers of Ag-experience) or enter the cycle (Fig. 6E–H) are unaffected in *Ppif*$^{-/-}$ mice, which suggests that priming of $CD8^+$ T cells is unaffected.

DCs are considered the major APC responsible for priming $CD8^+$ T cells. However, other APCs, including macrophages, have also been implicated in T cell responses (52, 56). In addition, macrophages have been suggested to contribute to memory $CD8^+$ T cell survival (59). Thus, we further analyzed the impact of CypD deficiency on macrophage populations. We first examined frequencies and numbers of macrophages in LCMV-Arm–infected *Ppif*$^{-/-}$ and WT mice on day 2 postinfection (macrophages were identified as $CD11b^+$, $F4/80^+$ cells). Although macrophage frequencies were similar (Fig. 7J, 7K), total numbers of macrophages were reduced in *Ppif*$^{-/-}$ mice compared with WT mice on day 2 post–LCMV-Arm infection (Fig. 7L, 1.9-fold). These results demonstrate that macrophage populations in the spleen are also impacted in *Ppif*$^{-/-}$ mice following LCMV-Arm infection.

WT macrophages can partially rescue CD8+ T cell impairment in Ppif$^{-/-}$ *mice*

Because our data suggest that DCs are activated normally in the absence of CypD and that the T cell defect is unlikely to be caused by a defect in T cell priming, we determined whether WT macrophages can provide signals to rescue optimal T cell responses in *Ppif*$^{-/-}$mice. Thus, we asked whether BMDM from WT mice were sufficient to rescue $CD8^+$ T cell numbers in LCMV-Arm–infected *Ppif*$^{-/-}$ mice. To test this, we derived and differentiated BMDM from *Ppif*$^{-/-}$ and WT mice and adoptively transferred them into *Ppif*$^{-/-}$–recipient mice. One day after transfer, recipient mice were infected with LCMV-Arm, and $CD8^+$ T cell numbers were examined on day 8 postinfection (Fig. 8A). In *Ppif*$^{-/-}$–recipient mice that received WT BMDM, we observed increased total spleen cellularity (Fig. 8B, 1.4-fold), increased bulk $CD8^+$ T cell populations (Fig. 8C, 1.7-fold), and increased Ag-experienced $CD8\alpha^{lo}$ $CD11a^{hi}$ $CD8^+$ T cells (Fig. 8D, 1.7-fold) compared with *Ppif*$^{-/-}$–recipient mice that received *Ppif*$^{-/-}$ BMDM. Further, we observed increased LCMV-specific D^b GP_{33-41}– and D^b $GP_{276-286}$–specific MHC class I tetramer$^+$ $CD8^+$ T cell numbers (Fig. 8E, 1.8-fold and Fig. 8F, 1.5-fold, respectively) and increased total numbers of IFN-γ and IFN-γ–TNF-α polyfunctional GP_{33-41}– and $GP_{276-286}$–specific $CD8^+$ T cells (Fig. 8G–J). Interestingly, although transfer of macrophages was

stimulation. (**I**) The total number of IFN-γ^+ TNF-α^+ $GP_{276-286}$–specific $CD8\alpha^+$ T cells in the spleen after $GP_{276-286}$ stimulation. (**J**) gMFI of TNF-α^+ $CD8\alpha^+$ T cells after $GP_{276-286}$ stimulation. Data analyzed by two-tailed, unpaired Student *t* test. Error bars represent SEM. Data are representative of two similar and independent experiments (three to five mice per group). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

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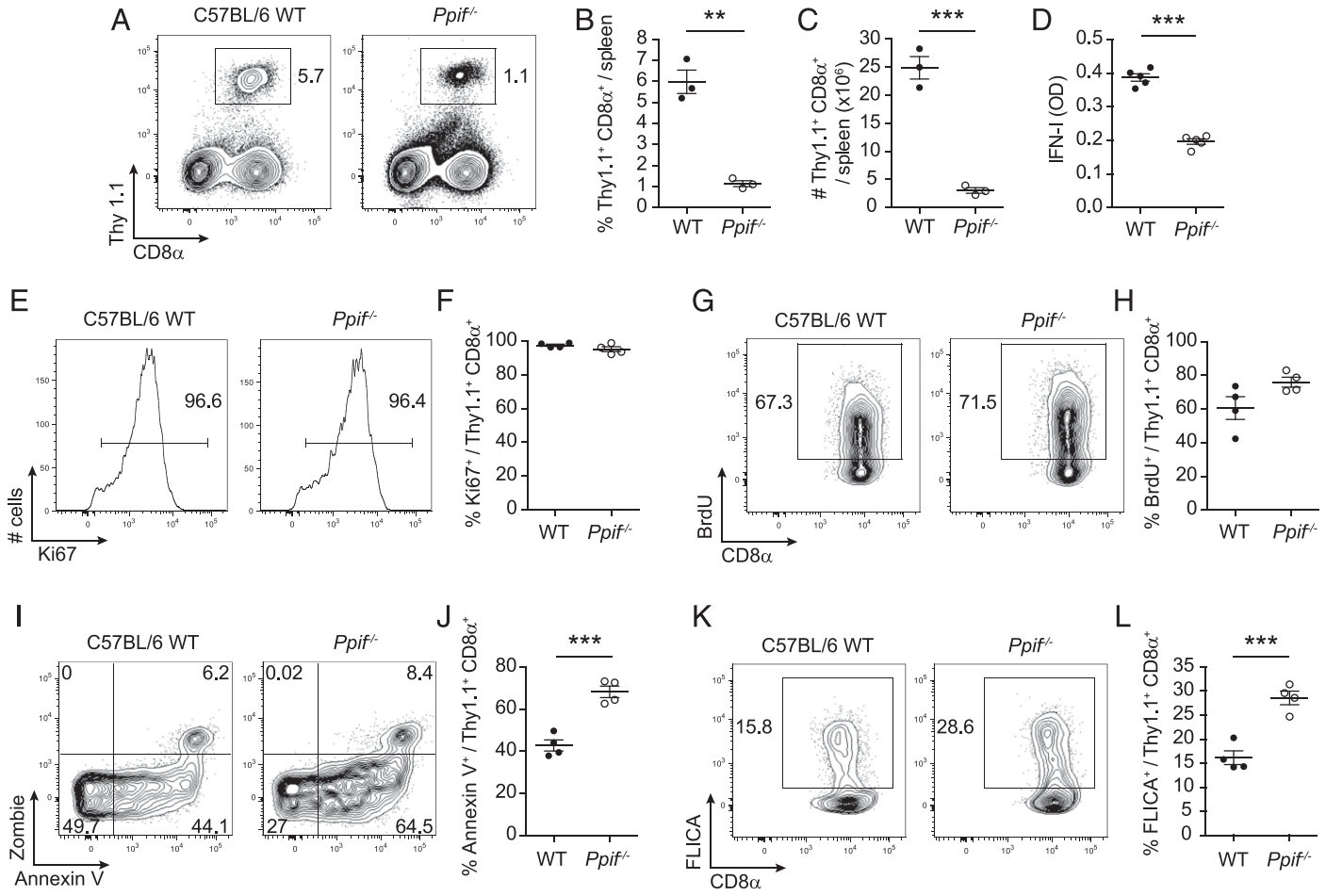

FIGURE 6. CypD deficiency impairs CD8+ T cell responses in a T cell–extrinsic manner, leading to decreased cell survival.
One day prior to infection with LCMV-Arm (2×10^5 PFU i.p.), 5000 WT P14 TCR–transgenic CD8α+ T cells (Thy1.1) were adoptively transferred into WT (Thy1.2) or *Ppif*$^{-/-}$ (Thy1.2)–recipient mice, and on day 8 postinfection, WT P14 CD8α+ T cell expansion was examined. (**A**) Representative flow cytometry plots of P14 (Thy1.1+ CD8α+) CD8α+ T cells present in the spleen. The frequency of donor WT P14 detected in the spleen on day 8 postinfection is indicated in each plot. (**B**) Frequency of WT P14 (Thy1.1+ CD8α+) CD8α+ T cells. (**C**) Total number of WT P14 (Thy1.1+ CD8α+) CD8α+ T cells in the spleen. (**D**) WT or *Ppif*$^{-/-}$ mice were infected with LCMV-Arm (2×10^5 PFU i.p.), and on day 3 postinfection, serum was collected. Total active IFN-I (IFN-α and IFN-β) serum levels were measured using B16-blue IFN-α/-β reporter cell line. Gated on WT P14 (Thy1.1+ CD8α+) CD8α+ T cells from splenocytes of WT or *Ppif*$^{-/-}$ day 8 post–LCMV-Arm–infected mice. (**E**) Illustrated are representative histograms of Ki67 expression. Frequency of Ki67+ WT P14 (Thy1.1+ CD8α+) CD8α+ T cells is indicated in each plot. (**F**) Frequency of Ki67+ WT P14 (Thy1.1+ CD8α+) CD8α+ T cells. (**G**) Illustrated are representative flow cytometry plots of BrdU expression. Frequency of BrdU+ WT P14 (Thy1.1+ CD8α+) CD8α+ T cells is indicated in each plot. (**H**) Frequency of BrdU+ WT P14 (Thy1.1+ CD8α+) CD8α+ T cells. (**I**) Representative flow cytometry of Zombie viability and Annexin V dye staining. Frequency of Zombie+ Annexin V+ WT P14 (Thy1.1+ CD8α+) CD8α+ T cells is indicated in each plot. (**J**) Frequency of Zombie− Annexin V+ WT P14 (Thy1.1+ CD8α+) CD8α+ T cells. (**K**) Representative flow cytometry of FLICA staining. Frequency of FLICA+ WT P14 (Thy1.1+ CD8α+) CD8α+ T cells is indicated in each plot. (**L**) Frequency of FLICA+ WT P14 (Thy1.1+ CD8α+) CD8α+ T cells. Data analyzed by two-tailed, unpaired Student *t* test. Error bars represent SEM. Data are representative of two similar and independent experiments (three to four mice per group). $^{**}p < 0.01$, $^{***}p < 0.001$.

sufficient to partially restore T cell numbers, it did not restore the expression of IFN-I (data not shown). Together, these results supported that CypD plays an extrinsic and critical role in regulating CD8+ T cell responses to LCMV-Arm infection and that expression of CypD by macrophages can provide signals that are important to, at least partially, restore CD8+ T cell responses in mice lacking CypD. However, this is independent of the role of CypD in regulating IFN-I expression.

DISCUSSION

Mitochondria play an important role in antiviral immunity, and activation of IFN-I signaling pathways are required for optimal antiviral CD8+ T cell responses. In this study, we showed that CypD, a modulatory component of the MPTP, plays a role in the induction of a CD8+ T cell–mediated antiviral immune response by promoting CD8+ T cell survival in a T cell–extrinsic

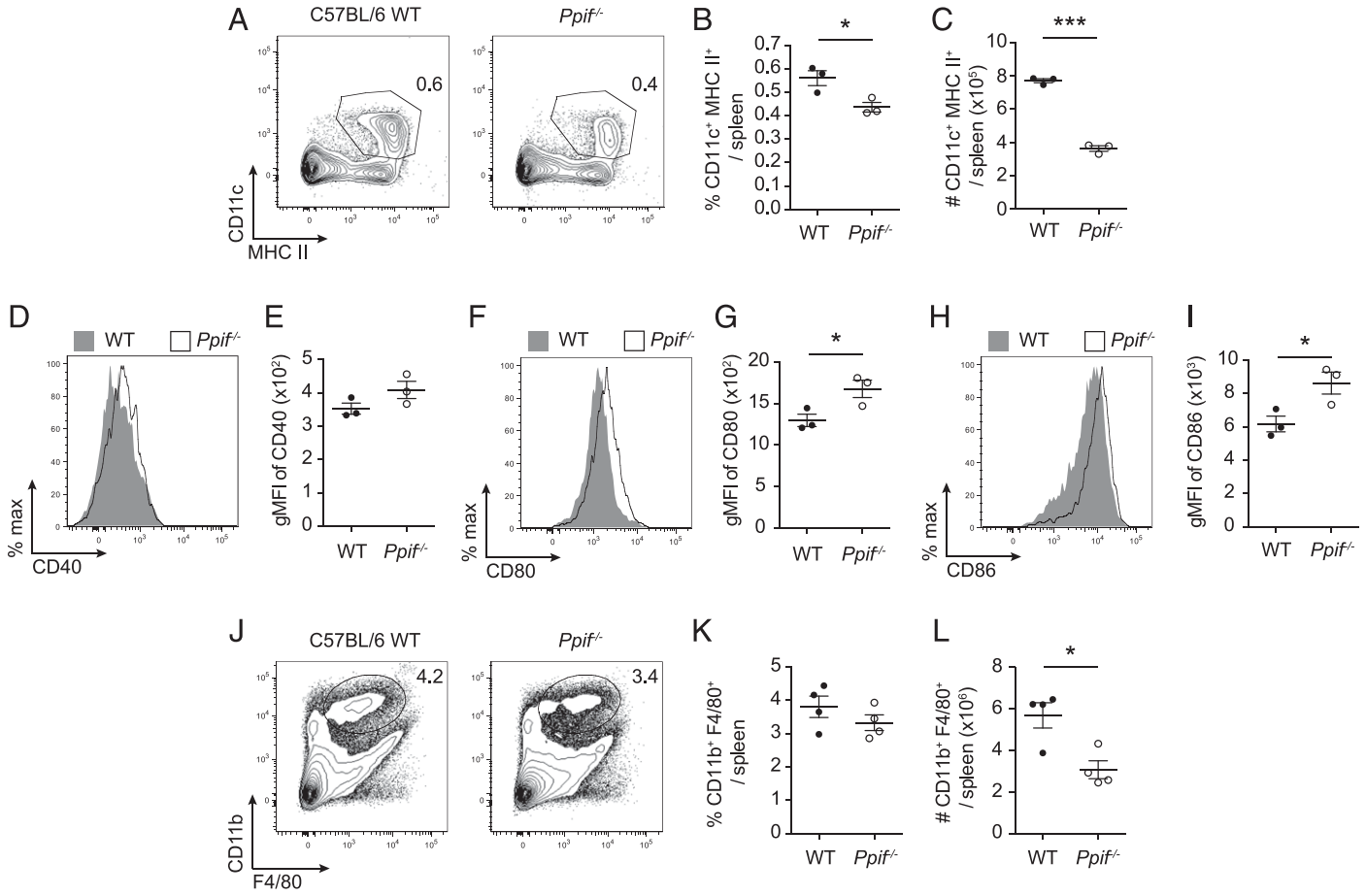

FIGURE 7. CypD deficiency impacts APCs.

WT or *Ppif*$^{-/-}$ mice were infected with LCMV-Arm (2×10^5 PFU i.p.), and on day 2 postinfection, DCs and macrophage analysis was performed. (**A**) Gated on CD3$^-$, CD19$^-$, and NK1.1$^-$, illustrated are representative flow cytometry plots of DCs (CD11c$^+$, MHCII$^+$) in the spleen. The frequency of DCs detected in the spleen is indicated in each plot. (**B**) The frequency and (**C**) total numbers of DCs (CD3$^-$, CD19$^-$, NK1.1$^-$, CD11c$^+$, MHCII$^+$) in the spleen. Gated on DCs (CD3$^-$, CD19$^-$, NK1.1$^-$, CD11c$^+$, MHCII$^+$). (**D**) Illustrated are representative histograms of CD40 expression. (**E**) gMFI of CD40 expression. (**F**) Representative histograms of CD80 expression. (**G**) gMFI of CD80 expression. (**H**) Representative histograms of CD86 expression. (**I**) gMFI of CD86 expression. WT shown in gray histogram and *Ppif*$^{-/-}$ shown in open histogram. (**J**) Representative flow cytometry plots of macrophages (CD11b$^+$ F4/80$^+$) present in the spleen. The frequency of macrophages detected in the spleen is indicated in each plot. (**K**) The frequency and (**L**) Total numbers of macrophages (CD11b$^+$, F4/80$^+$) in the spleen. Data analyzed by two-tailed, unpaired Student *t* test. Error bars represent SEM. Data are representative of two similar and independent experiments (three to five mice per group). *$p < 0.05$, ***$p < 0.001$.

manner. In addition, our results suggest that CypD plays a role in the induction of IFN-I but that this is likely independent of its role in maintaining CD8$^+$ T cell survival. Reduced IFN-I or reduction in the number of innate cells such as macrophages and DCs in the absence of CypD may play a role in reducing the early capacity of the host to control pathogen burden, as we observed increased viral load at an early time-point following infection. Thus, we have identified a novel role for CypD as an important player in antiviral immunity, which can influence both the production of IFN-I and the induction of an optimal CD8$^+$ T cell response.

CypD plays a role in macrophage immunity to *M. tuberculosis* infection, in which CypD inhibition with cyclosporine A results in decreased in vitro pathogen growth and necrosis (60). Despite this, it has been recently shown that following *M. tuberculosis* infection, CypD-deficient mice have enhanced T cell responses that contribute to significant lung tissue damage and increased susceptibility to infection, without modulating in vivo pathogen loads. Furthermore, it was demonstrated that CypD intrinsically regulates T cell responses to *M. tuberculosis* infection (6). In this study, we showed that lack of CypD resulted in decreased CD8$^+$ T cell responses to LCMV-Arm infection that is attributed, at least in part, to T cell–extrinsic factors. The fact that CypD intrinsically inhibits detrimental, overly exuberant T cell responses to *M. tuberculosis* while extrinsically promoting protective T cell responses to LCMV infection may have important underlying biological mechanisms. *M. tuberculosis* is a chronic bacterial infection that induces a delayed adaptive response (weeks postinfection) that persists,

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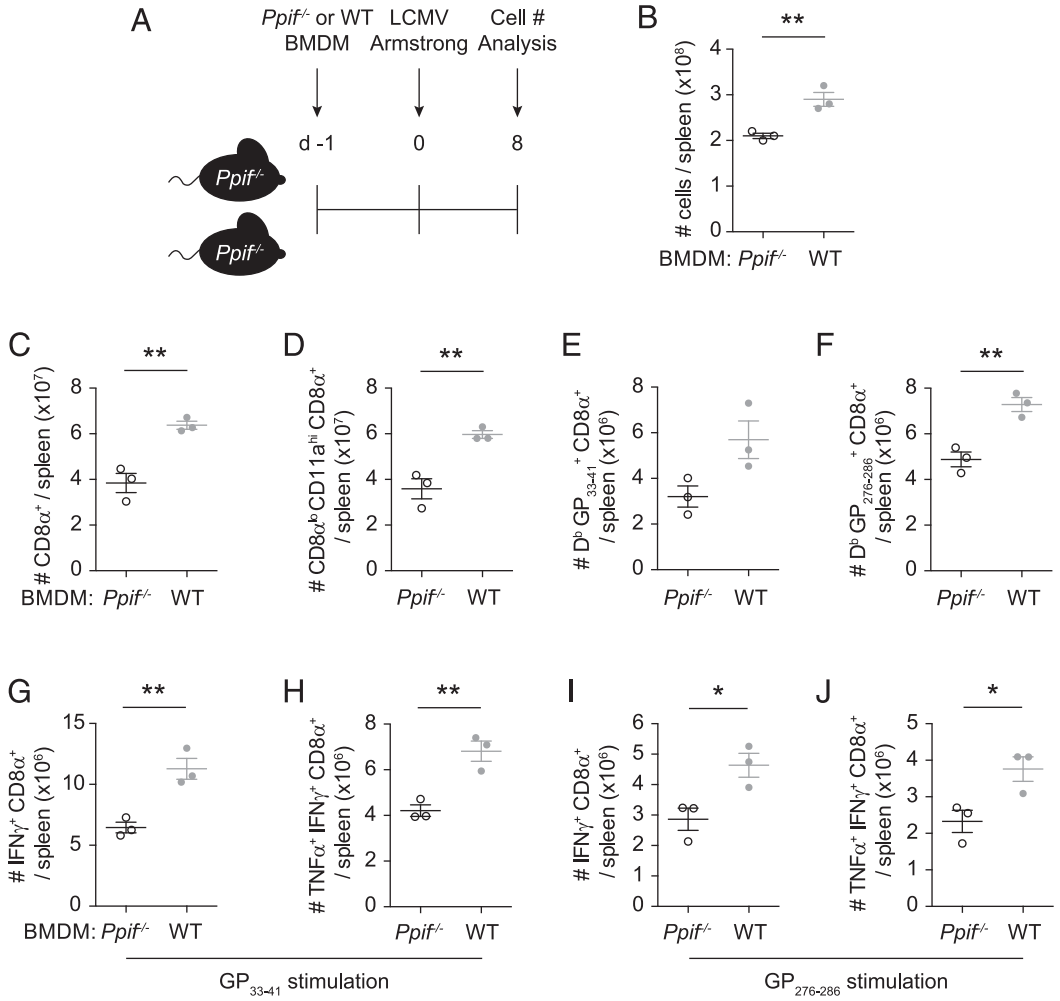

FIGURE 8. CypD expression in macrophages can partially rescue CD8$^+$ T cell responses.

(**A**) Experimental design is as follows: BMDMs were derived and differentiated from naive WT and *Ppif*$^{-/-}$ mice, and 1×10^6 BMDMs were adoptively transferred into *Ppif*$^{-/-}$–recipient mice. The following day, recipient mice were infected with LCMV-Arm (2×10^5 PFU i.p.), and on day 8 postinfection, CD8$^+$ T cell numbers were examined. (**B**) Total spleen cellularity. (**C**) The number of bulk CD8α$^+$ T cell polyclonal population in the spleen. (**D**) Total number of CD8α^{lo} CD11a^{hi} Ag-experienced CD8α$^+$ T cells in the spleen. (**E**) Total number of MHC class I H-2D^b tetramer D^b GP$_{33-41}$–specific and (**F**) D^b GP$_{276-286}$–specific CD8α$^+$ T cells in the spleen. (**G**) Total number of IFN-γ$^+$ GP$_{33-41}$–specific and (**H**) IFN-γ$^+$ TNF-α$^+$ GP$_{33-41}$–specific CD8α$^+$ T cells in the spleen. (**I**) Total number of IFN-γ$^+$ GP$_{276-286}$–specific and (**J**) IFN-γ$^+$ TNF-α$^+$ GP$_{276-286}$–specific CD8α$^+$ T cells in the spleen. Data analyzed by two-tailed, unpaired Student *t* test. Error bars represent SEM. Data are representative of two similar and independent experiments (three to five mice per group). *$p < 0.05$, **$p < 0.01$.

such that regulation of T cell proliferation versus contraction is essential for the maintenance of granuloma and prevention of *M. tuberculosis* dissemination. In contrast, LCMV-Arm infects acutely and promotes a robust T cell response (days postinfection) that contributes directly to viral killing. Thus, it may be that CypD has differential roles in response to bacterial versus viral infections. Furthermore, the role of CypD may differ between macrophages and T cells. Of note, although our study clearly identified a novel T cell–extrinsic role for CypD in inducing optimal T cell responses, our data do not rule out a potential T cell–intrinsic role for CypD in the context of viral infection. These interesting questions will be the subject of future investigations.

Importantly, our findings suggested that macrophages may be one of the major cell types responsible for the reduced CD8$^+$ T cell response in *Ppif*$^{-/-}$ mice, as adoptive transfer of WT macrophages into LCMV-Arm–infected CypD-deficient hosts enhanced Ag-specific CD8$^+$ T cell numbers. Interestingly, WT macrophages were able to increase CD8$^+$ T cell numbers without restoring the IFN-I response in *Ppif*$^{-/-}$ mice, which is an important signal to maintain CD8$^+$ T cell proliferation (16–21, 23). This is in line with our data demonstrating that the reduced number of Ag-specific CD8$^+$ T cells in *Ppif*$^{-/-}$ mice is not because of a defect in cell cycle entry or proliferation but rather to an increase in CD8$^+$ T cell death. This suggests the intriguing concept that macrophages may be

important to maintain effector CD8+ T cell viability in a CypD-dependent manner. Previous studies have suggested that macrophages provide key signals to maintain viability of memory CD8+ T cells (59). As such, it is tempting to speculate that macrophages may be providing key survival signals during the course of effector CD8+ T cell responses as well and that this is dependent on the expression of CypD. Thus, CypD expression by macrophages may be important for the induction of optimal CD8+ T cell responses, whereas CypD expression by other cell types (potentially including T cells) is important for the regulation of IFN-I. However, the adoptive transfer approach used in this study does not necessarily demonstrate that CypD expression by macrophages is absolutely required for optimal CD8+ T cell responses. Rather, it suggests that WT macrophages are at least able to provide some of the signals that are lacking in CypD-deficient mice to sustain CD8+ T cell survival. The macrophage-intrinsic role of CypD in maintaining CD8+ T cell survival will be the focus of future studies. In addition, it is important to note that transfer of macrophages was not sufficient to fully restore T cell numbers to WT levels. This may simply be a result of the low number of macrophages transferred that yields an incomplete rescue but may also suggest that CypD expression by other cell types is important for optimal CD8+ T cell accumulation.

In summary, we have shown a novel role for CypD in CD8+ T cell–mediated responses to viral infection. CypD plays a role in the MPTP and cell death, and drugs that inhibit its function such as cyclosporine A have been used in patients with liver, heart, and brain injuries (5). In this study, we demonstrated that CypD deficiency affected the innate antiviral response, which in turn is associated with marked impairments in CD8+ T cell survival and accumulation following acute viral infection in mice. Thus, caution is warranted when administering CypD-inhibitory drugs, as host immunity to certain viruses may be compromised.

DISCLOSURES

The authors have no financial conflicts of interest.

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REFERENCES

1. Baines, C. P., R. A. Kaiser, N. H. Purcell, N. S. Blair, H. Osinska, M. A. Hambleton, E. W. Brunskill, M. R. Sayen, R. A. Gottlieb, G. W. Dorn, et al. 2005. Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death. *Nature* 434: 658–662.
2. Nakagawa, T., S. Shimizu, T. Watanabe, O. Yamaguchi, K. Otsu, H. Yamagata, H. Inohara, T. Kubo, and Y. Tsujimoto. 2005. Cyclophilin D-dependent mitochondrial permeability transition regulates some necrotic but not apoptotic cell death. *Nature* 434: 652–658.
3. Wang, P., and J. Heitman. 2005. The cyclophilins. *Genome Biol.* 6: 226.
4. Elrod, J. W., and J. D. Molkentin. 2013. Physiologic functions of cyclophilin D and the mitochondrial permeability transition pore. *Circ. J.* 77: 1111–1122.
5. Halestrap, A. 2005. Biochemistry: a pore way to die. *Nature* 434: 578–579.
6. Tzelepis, F., J. Blagih, N. Khan, J. Gillard, L. Mendonca, D. G. Roy, E. H. Ma, P. Joubert, R. G. Jones, and M. Divangahi. 2018. Mitochondrial cyclophilin D regulates T cell metabolic responses and disease tolerance to tuberculosis. *Sci. Immunol.* 3: eaar4135.
7. Zinkernagel, R. M., and P. C. Doherty. 1974. Restriction of in vitro T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. *Nature* 248: 701–702.
8. Ada, G. 1994. Twenty years into the saga of MHC-restriction. *Immunol. Cell Biol.* 72: 447–454.
9. Zinkernagel, R. M., and P. C. Doherty. 1997. The discovery of MHC restriction. *Immunol. Today* 18: 14–17.
10. Lanzavecchia, A., and F. Sallusto. 2001. Antigen decoding by T lymphocytes: from synapses to fate determination. *Nat. Immunol.* 2: 487–492.
11. Mempel, T. R., S. E. Henrickson, and U. H. Von Andrian. 2004. T-cell priming by dendritic cells in lymph nodes occurs in three distinct phases. *Nature* 427: 154–159.
12. Boise, L. H., A. J. Minn, P. J. Noel, C. H. June, M. A. Accavitti, T. Lindsten, and C. B. Thompson. 1995. CD28 costimulation can promote T cell survival by enhancing the expression of Bcl-XL. *Immunity* 3: 87–98.
13. Carreno, B. M., and M. Collins. 2002. The B7 family of ligands and its receptors: new pathways for costimulation and inhibition of immune responses. *Annu. Rev. Immunol.* 20: 29–53.
14. Fujii, S., K. Liu, C. Smith, A. J. Bonito, and R. M. Steinman. 2004. The linkage of innate to adaptive immunity via maturing dendritic cells in vivo requires CD40 ligation in addition to antigen presentation and CD80/86 costimulation. *J. Exp. Med.* 199: 1607–1618.
15. Eberlein, J., B. Davenport, T. T. Nguyen, F. Victorino, T. Sparwasser, and D. Homann. 2012. Multiple layers of CD80/86-dependent costimulatory activity regulate primary, memory, and secondary lymphocytic choriomeningitis virus-specific T cell immunity. *J. Virol.* 86: 1955–1970.
16. Mescher, M. F., J. M. Curtsinger, P. Agarwal, K. A. Casey, M. Gerner, C. D. Hammerbeck, F. Popescu, and Z. Xiao. 2006. Signals required for programming effector and memory development by CD8+ T cells. *Immunol. Rev.* 211: 81–92.
17. Curtsinger, J. M., and M. F. Mescher. 2010. Inflammatory cytokines as a third signal for T cell activation. *Curr. Opin. Immunol.* 22: 333–340.
18. Butler, N. S., J. C. Nolz, and J. T. Harty. 2011. Immunologic considerations for generating memory CD8 T cells through vaccination. *Cell. Microbiol.* 13: 925–933.
19. Richer, M. J., J. C. Nolz, and J. T. Harty. 2013. Pathogen-specific inflammatory milieux tune the antigen sensitivity of CD8(+) T cells by enhancing T cell receptor signaling. *Immunity* 38: 140–152.
20. Starbeck-Miller, G. R., H. H. Xue, and J. T. Harty. 2014. IL-12 and type I interferon prolong the division of activated CD8 T cells by maintaining high-affinity IL-2 signaling in vivo. *J. Exp. Med.* 211: 105–120.
21. Richer, M. J., L. L. Pewe, L. S. Hancox, S. M. Hartwig, S. M. Varga, and J. T. Harty. 2015. Inflammatory IL-15 is required for optimal memory T cell responses. *J. Clin. Invest.* 125: 3477–3490.
22. Valbon, S. F., S. A. Condotta, and M. J. Richer. 2016. Regulation of effector and memory CD8(+) T cell function by inflammatory cytokines. *Cytokine* 82: 16–23.
23. Kolumam, G. A., S. Thomas, L. J. Thompson, J. Sprent, and K. Murali-Krishna. 2005. Type I interferons act directly on CD8 T cells to allow clonal expansion and memory formation in response to viral infection. *J. Exp. Med.* 202: 637–650.

24. Oldstone, M. B., and F. J. Dixon. 1970. Tissue injury in lymphocytic choriomeningitis viral infection: virus-induced immunologically specific release of a cytotoxic factor from immune lymphoid cells. *Virology* 42: 805–813.
25. Marker, O., and M. Volkert. 1973. Studies on cell-mediated immunity to lymphocytic choriomeningitis virus in mice. *J. Exp. Med.* 137: 1511–1525.
26. Doherty, P. C., and R. M. Zinkernagel. 1975. H-2 compatibility is required for T-cell-mediated lysis of target cells infected with lymphocytic choriomeningitis virus. *J. Exp. Med.* 141: 502–507.
27. Kägi, D., B. Ledermann, K. Bürki, P. Seiler, B. Odermatt, K. J. Olsen, E. R. Podack, R. M. Zinkernagel, and H. Hengartner. 1994. Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. *Nature* 369: 31–37.
28. Ahmed, R., and D. Gray. 1996. Immunological memory and protective immunity: understanding their relation. *Science* 272: 54–60.
29. Butz, E. A., and M. J. Bevan. 1998. Massive expansion of antigen-specific CD8+ T cells during an acute virus infection. *Immunity* 8: 167–175.
30. Murali-Krishna, K., J. D. Altman, M. Suresh, D. J. Sourdive, A. J. Zajac, J. D. Miller, J. Slansky, and R. Ahmed. 1998. Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection. *Immunity* 8: 177–187.
31. Kaech, S. M., E. J. Wherry, and R. Ahmed. 2002. Effector and memory T-cell differentiation: implications for vaccine development. *Nat. Rev. Immunol.* 2: 251–262.
32. Badovinac, V. P., J. S. Haring, and J. T. Harty. 2007. Initial T cell receptor transgenic cell precursor frequency dictates critical aspects of the CD8(+) T cell response to infection. *Immunity* 26: 827–841.
33. Pircher, H., J. Baenziger, M. Schilham, T. Sado, H. Kamisaku, H. Hengartner, and R. M. Zinkernagel. 1987. Characterization of virus-specific cytotoxic T cell clones from allogeneic bone marrow chimeras. *Eur. J. Immunol.* 17: 159–166.
34. Welsh, R. M., and M. O. Seedhom. 2008. Lymphocytic choriomeningitis virus (LCMV): propagation, quantitation, and storage. *Curr. Protoc. Microbiol.* Chapter 15: Unit 15A.1.
35. Condotta, S. A., S. H. Khan, D. Rai, T. S. Griffith, and V. P. Badovinac. 2015. Polymicrobial sepsis increases susceptibility to chronic viral infection and exacerbates CD8+ T cell exhaustion. *J. Immunol.* 195: 116–125.
36. Altman, J. D., P. A. Moss, P. J. Goulder, D. H. Barouch, M. G. McHeyzer-Williams, J. I. Bell, A. J. McMichael, and M. M. Davis. 1996. Phenotypic analysis of antigen-specific T lymphocytes. *Science* 274: 94–96.
37. Rai, D., N. L. Pham, J. T. Harty, and V. P. Badovinac. 2009. Tracking the total CD8 T cell response to infection reveals substantial discordance in magnitude and kinetics between inbred and outbred hosts. *J. Immunol.* 183: 7672–7681.
38. Pardy, R. D., M. M. Rajah, S. A. Condotta, N. G. Taylor, S. M. Sagan, and M. J. Richer. 2017. Analysis of the T cell response to Zika virus and identification of a novel CD8+ T cell epitope in immunocompetent mice. *PLoS Pathog.* 13: e1006184.
39. Zhou, X., S. Ramachandran, M. Mann, and D. L. Popkin. 2012. Role of lymphocytic choriomeningitis virus (LCMV) in understanding viral immunology: past, present and future. *Viruses* 4: 2650–2669.
40. Althaus, C. L., V. V. Ganusov, and R. J. De Boer. 2007. Dynamics of CD8+ T cell responses during acute and chronic lymphocytic choriomeningitis virus infection. *J. Immunol.* 179: 2944–2951.
41. Homann, D., L. Teyton, and M. B. Oldstone. 2001. Differential regulation of antiviral T-cell immunity results in stable CD8+ but declining CD4+ T-cell memory. *Nat. Med.* 7: 913–919.
42. Wherry, E. J., J. N. Blattman, K. Murali-Krishna, R. van der Most, and R. Ahmed. 2003. Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. *J. Virol.* 77: 4911–4927.
43. Kotturi, M. F., B. Peters, F. Buendia-Laysa Jr., J. Sidney, C. Oseroff, J. Botten, H. Grey, M. J. Buchmeier, and A. Sette. 2007. The CD8+ T-cell response to lymphocytic choriomeningitis virus involves the L antigen: uncovering new tricks for an old virus. *J. Virol.* 81: 4928–4940.
44. Raué, H. P., and M. K. Slifka. 2009. CD8+ T cell immunodominance shifts during the early stages of acute LCMV infection independently from functional avidity maturation. *Virology* 390: 197–204.
45. McDermott, D. S., and S. M. Varga. 2011. Quantifying antigen-specific CD4 T cells during a viral infection: CD4 T cell responses are larger than we think. *J. Immunol.* 187: 5568–5576.
46. Butler, N. S., J. Moebius, L. L. Pewe, B. Traore, O. K. Doumbo, L. T. Tygrett, T. J. Waldschmidt, P. D. Crompton, and J. T. Harty. 2011. Therapeutic blockade of PD-L1 and LAG-3 rapidly clears established blood-stage *Plasmodium* infection. *Nat. Immunol.* 13: 188–195.
47. Seder, R. A., P. A. Darrah, and M. Roederer. 2008. T-cell quality in memory and protection: implications for vaccine design. [Published erratum appears in 2008 *Nat. Rev. Immunol.* 8: 486.] *Nat. Rev. Immunol.* 8: 247–258.
48. Han, Q., N. Bagheri, E. M. Bradshaw, D. A. Hafler, D. A. Lauffenburger, and J. C. Love. 2012. Polyfunctional responses by human T cells result from sequential release of cytokines. *Proc. Natl. Acad. Sci. USA* 109: 1607–1612.
49. Duvall, M. G., M. L. Precopio, D. A. Ambrozak, A. Jaye, A. J. McMichael, H. C. Whittle, M. Roederer, S. L. Rowland-Jones, and R. A. Koup. 2008. Polyfunctional T cell responses are a hallmark of HIV-2 infection. *Eur. J. Immunol.* 38: 350–363.
50. Ciuffreda, D., D. Comte, M. Cavassini, E. Giostra, L. Bühler, M. Perruchoud, M. H. Heim, M. Battegay, D. Genné, B. Mulhaupt, et al. 2008. Polyfunctional HCV-specific T-cell responses are associated with effective control of HCV replication. *Eur. J. Immunol.* 38: 2665–2677.
51. Casetti, R., G. De Simone, A. Sacchi, V. Bordoni, D. Viola, A. Rinaldi, C. Agrati, C. Gioia, and F. Martini. 2014. Modulation of polyfunctional HIV-specific CD8 T cells in patients responding differently to antiretroviral therapy. *Int. J. Immunopathol. Pharmacol.* 27: 291–297.
52. Hilpert, C., S. Sitte, A. Matthies, and D. Voehringer. 2016. Dendritic cells are dispensable for T cell priming and control of acute lymphocytic choriomeningitis virus infection. *J. Immunol.* 197: 2780–2786.
53. Banchereau, J., F. Bazan, D. Blanchard, F. Brière, J. P. Galizzi, C. van Kooten, Y. J. Liu, F. Rousset, and S. Saeland. 1994. The CD40 antigen and its ligand. *Annu. Rev. Immunol.* 12: 881–922.
54. Vasilevko, V., A. Ghochikyan, M. J. Holterman, and M. G. Agadjanyan. 2002. CD80 (B7-1) and CD86 (B7-2) are functionally equivalent in the initiation and maintenance of CD4+ T-cell proliferation after activation with suboptimal doses of PHA. *DNA Cell Biol.* 21: 137–149.
55. Sansom, D. M., C. N. Manzotti, and Y. Zheng. 2003. What's the difference between CD80 and CD86? *Trends Immunol.* 24: 314–319.
56. Tan, J. K., and H. C. O'Neill. 2005. Maturation requirements for dendritic cells in T cell stimulation leading to tolerance versus immunity. *J. Leukoc. Biol.* 78: 319–324.
57. Elgueta, R., M. J. Benson, V. C. de Vries, A. Wasiuk, Y. Guo, and R. J. Noelle. 2009. Molecular mechanism and function of CD40/CD40L engagement in the immune system. *Immunol. Rev.* 229: 152–172.
58. Ma, D. Y., and E. A. Clark. 2009. The role of CD40 and CD154/CD40L in dendritic cells. *Semin. Immunol.* 21: 265–272.
59. Frasca, L., S. W. Stonier, W. W. Overwijk, and K. S. Schluns. 2010. Differential mechanisms of memory CD8 T cell maintenance by individual myeloid cell types. *J. Leukoc. Biol.* 88: 69–78.
60. Gan, H., X. He, L. Duan, E. Mirabile-Levens, H. Kornfeld, and H. G. Remold. 2005. Enhancement of antimycobacterial activity of macrophages by stabilization of inner mitochondrial membrane potential. *J. Infect. Dis.* 191: 1292–1300.